FUNCTIONAL PROFILING OF SINGLE-NUCLEOTIDE POLYMORPHISMS ASSOCIATED WITH BIPOLAR DISORDER

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1 PREFACE

1.1 ACKNOWLEDGEMENTS

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“endele dreit føle”

1.2 ABBREVIATIONS

BOLD	Blood-Oxygen-Level-Dependent
BD	Bipolar Disorder
CNV	Copy Number Variant
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th edition
eQTl	Expressed Quantitative Trait Loci
GO	Gene Ontology
GWAS	Genome-Wide Association Study
ICD-10	International Classification of Disease, 10th revision
Kbp	Kilo-base-pairs or 1000 base pairs of DNA/RNA
LCT	Lactase
LD	Linkage disequilibrium
MRI	Magnetic Resonance Imaging
OR	Odds Ratio
PCA	Principal Component Analysais
PGC	Psychiatric GWAS Consortium
PPI	Protein-protein interaction
QC	Quality Control
ROI	Region of Interest
SCZ	Schizophrenia
SNP	Single Nucleotide Polymorphism
TOP	Thematically Organized Psychosis study
WTCCC	Welcome Trust Case Control Consortium

1.3 LIST OF PAPERS

Paper I

Linkage-disequilibrium-based binning affects the interpretation of GWASs.

Christoforou A, Dondrup M, Mattingsdal M, Mattheisen M, Giddaluru S, Nöthen MM, Rietschel M, Cichon S, Djurovic S, Andreassen OA, Jonassen I, Steen VM, Puntervoll P, Le Hellard S.

Am J Hum Genet. 2012 Apr 6;90(4):727-33

Paper II

Pathway analysis of genetic markers associated with a functional MRI faces paradigm implicates polymorphisms in calcium responsive pathways.

Mattingsdal M, Brown AA, Djurovic S, Sønderby IE, Server A, Melle I, Agartz I, Hovig E, Jensen J, Andreassen OA.

Neuroimage. 2013 Apr 15;70:143-9.

Paper III

Network-based gene set enrichment of genomic regions associated with bipolar disorder reveals susceptibility genes responsive to oestrogen stimulus.

Mattingsdal M, Wang Y, Thompson WK, Hovig E, Djurovic S, Dale AM, Andreassen OA

Manuscript.

1.3.1 ASSOCIATED PAPERS

Gene variants associated with schizophrenia in a Norwegian genome-wide study are replicated in a large European cohort. Athanasiu L, Mattingsdal M, Kähler AK, Brown A, Gustafsson O, Agartz I, Giegling I, Muglia P, Cichon S, Rietschel M, Pietiläinen OP, Peltonen L, Bramon E, Collier D, Clair DS, Sigurdsson E, Petursson H, Rujescu D, Melle I, Steen VM, Djurovic S, Andreassen OA. *J Psychiatr Res. 2010 Sep;44(12):748-53. Epub 2010 Feb 24.*

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Identification of common variants associated with human hippocampal and intracranial volumes. Enhancing Neuro Imaging Genetics through Meta-Analysis Consortium. *Nat Genet. 2012 Apr 15;44(5):552-61. doi: 10.1038/ng.22*

2 ABSTRACT

Bipolar disorder (BD) is a condition with high heritability estimates, suggesting the influence of biological determinants. However, identifying the nature of these biological determinants has proven to be difficult, even after decades of genetic research. Numerous hypotheses exist, and correspondingly, a substantial amount of heterogeneous literature claims significant findings. With increasing technological advances, larger experiments are being performed both in terms of number of subjects included in the studies, as well as measuring genetic elements. This includes genome-wide association studies (GWAS), measuring ancient point mutations in DNA distributed within a population, also known as Single Nucleotide Polymorphisms (SNPs), and statistically testing them for association to a trait of interest. The scale of modern biological experiments is truly massive and requires appropriate statistical tools, in order to identify statistically significant findings. Calculating p-values from empirical data using classical statistical methods is relatively straightforward. However, interpreting the findings into a molecular and biological context is not straightforward. Publically available data like the genetic architecture across populations, knowledge of gene and protein functions may be integrated and can aid interpretation of a large number of SNPs, returned from GWAS. Grouping genes together into gene sets based on knowledge and statistical testing if any gene sets significantly overlap with SNPs and genes associated through GWAS is known as gene set analysis, and applications in BD generic research is the main focus of this work.

The first step in gene set analysis is to assign SNPs to genes. This is non-trivial as SNPs tend to be located outside the physical boundaries of genes, or located within a gene but influences the expression on a neighbouring gene. We show that genetic distance (LD) together with physical distance, and not physical distance alone, improves SNP to gene assignment, measured by an increase in concordance between studies (paper I). SNPs may influence brain function and activity, and there is a large interest in identifying characteristic neural patterns in BD. Collecting large brain imaging samples are labour intensive and methods for improving statistical power are warranted. Gene set analysis improves statistical power by detecting recurring associations in related genes. We conduct gene set analysis of moderately associated SNPs in a GWAS of an fMRI experiment, and find biological meaningful,

but not statistical significant findings (paper II). Genes carry information of how gene products shall interact with the cellular environment. We use protein-protein interaction data as a contextual filter to test if any genomic regions associated with BD contain genes encoding proteins which participate in a common biological process, possibly revealing a molecular context in which BD related proteins participate. We identify a biological process, genes responsive to oestrogen stimulus, as significantly over-represented among BD associated genes (paper III).

2.1 NORWEGIAN ABSTRACT

Bipolar lidelse har høy arvbarhet som antyder genetisk påvirkning. Men selv etter flere tiår med genetisk forskning, er de underliggende genetiske faktorene som bidrar til å danne sårbarhet for å utvikle bipolar lidelse fremdeles ukjent. Flere hypoteser finnes, og en betraktelig og sammensatt vitenskapelig litteratur rapporterer om mulige funn. Selv om likheten i DNA mellom mennesker er høy, er det relativt lett å finne forskjeller. Spesielt enkeltmutasjoner er vanlige. De fleste enkeltmutasjonene mellom mennesker er nedarvet og har et urgammelt opphav og kalles SNPs. Disse gamle og nedarvete SNPs bidrar stekt til variasjonen vi kan observere mellom mennesker i dag, slik som variasjon i høyde, pigmentering og sårbarhet for å utvikle komplekse sykdommer som for eksempel auto immune sykdommer eller psykiske lidelser. Teknologiske fremskritt har gjort det mulig å effektivt måle frekvensen av disse SNPs i en populasjon, slik at man kan sammenligne frekvensen mellom en kontroll populasjon og en syk populasjon. Slik kan man statistisk teste om det er noen SNPs og gener som er signifikant assosiert med sykdom, og dermed forstå den underliggende biologien bedre. Slike studier kalles “Genome-Wide Association Studies” eller GWAS. Å estimere statistisk signifikante assosiasjoner vha. klassiske statistiske metoder er relativt enkelt. Men det å tolke funnene er ikke fullt så enkelt. Offentlig tilgjengelig data om gen-funksjon, gen-uttrykk og gen-interaksjoner kan brukes til å forbedre tolkningen av GWAS resultater. Å gruppere gener sammen, basert på kunnskap om gen funksjon, for så å teste om grupper av gener er over-representert i GWAS resultater kalles gen-sett analyse, og anvendelser innen genetisk forskning innen bipolar lidelse er hovedfokuset i denne avhandlingen.

GWAS returnerer SNPs, og ikke gener. Derfor er det første steget innen gen-sett analyse av GWAS resultater å tildele SNPs til gener. Dette er ikke enkelt siden SNPs ofte er plassert mellom gener, eller er plassert inni de fysiske grensene til ett gen, men påvirker nabogenet. Vi viser at genetisk avstand (LD) sammen med fysisk avstand, forbedrer tildelingen av SNPs til gener, målt ved økt konkordans mellom studier (artikkel 1). Effekten av SNPs på hjernefunksjon er lite kjent. Årsaken til dette er at nåværende teknikker for å måle hjerneaktivitet (fMRI) er omfattende og tidkrevende. Dette fører til at det er vanskelig å samle store nok materialer slik at man har statistisk styrke til å finne signifikante SNPs som påvirker hjerneaktivitet. Ett alternativ er å øke statistisk styrke andre steder i analysen. I denne studien (artikkel

II) har vi økt statistisk styrke ved å redusere datamengden av målt hjerneaktivitet til nye variabler, og brukt disse nye variablene i GWAS, som vi tolker vha. gen-sett analyse. I denne studien klarte vi ikke å finne noen statistisk signifikante funn. Gener koder for informasjon om hvordan gen produktet skal interagere med det molekylære miljøet. Vi bruker kjente protein interaksjoner som et logisk kontekst filter, for så å teste om det er noen gen-sett som er overrepresentert mellom gener assosiert med bipolar lidelse. I denne studien finner vi ett signifikant funn, at bipolar assosierte gener som interagerer med hverandre er beriket med proteiner som aktiveres av estrogen.

3 INTRODUCTION

3.1 SEVERE MENTAL DISORDERS

Severe mental disorders and the state of psychosis can manifest symptoms in human behaviour that are bizarre, disturbing and difficult for bystanders and even professionals to fully comprehend. Historically, severe mental disorders and accompanying psychotic episodes have often been met with superstition and fear, sometimes resulting in physical abuse and outright torture, attempting to banish possessing daemons. Even in modern times, so-called lunatics were housed in large institutional asylums sometimes being subjected to well-intended, but failed treatments, like lobotomies and insulin coma therapies. A shift occurred in the understanding of mental disorders and bipolar disorder (BD) in the beginning of the 1950ties by the discovery of mood stabilizing, psychoactive and antipsychotic drugs, able to chemically induce and inhibit changes in consciousness, mood, behaviour, and perception. Although the treatment and general clinical approach to BD are vastly more dignified, many consider the prognosis and current treatment unsatisfactory indicated by unexplained heterogeneous outcome and adverse effects of current medications. The biological influences in these disorders are not well understood and a substantial degree of personal suffering, socioeconomic burden and social stigma remains. WHO estimates that all mental disorders combined surpass both cancer and cardiovascular disease, when measuring disability-adjusted life years of the non-communicable diseases. The present etiological paradigm revolves around the classical Diathesis-stress model, where stressful life events together with biological predisposition, form vulnerability to these disorders. Although several stressful life events including physical, sexual and illegal substance abuse at young age are well known risk factors, the largest risk factor is having a first degree relative with BD, strongly indicating that BD is a heritable condition. However, the biological understanding and well founded knowledge about the underlying genetic predisposition of BD at possible dysfunctional biological processes is almost completely unknown.

3.2 A VERY BRIEF HISTORY OF BIPOLAR DISORDER

The German psychiatrist and one of the founders of modern psychiatry, Emil Kraepelin, pioneered the nosological classification of severe mental disorders and

psychosis, and in 1893 defined two distinct forms: dementia praecox and manic depression, later known as the Kraepelinian dichotomy and schizophrenia and bipolar disorder. Subsequent nosological effort further split the manic depressed category into unipolar disorder and bipolar disorder, by the Wernicke-Kleist-Leonhard school (1953), and later a distinction was made in bipolar disorder, between depressions with mania and depression with hypomania (1971), later known as bipolar type I (BD I) and bipolar disorder type II (BD II), reviewed [1]. The nature of the manic state continues to define the two main categories of BD, where mania which may include psychotic episodes, while hypomania is considered a milder form of mania and the absence of psychosis.

There are no experimental tests to empirically determine the presence of BD and the field of psychiatry remain a descriptive science, where observations of behaviour and communication of mental states, feelings and experiences are central in determining diagnosis. Misclassification is not uncommon, and it is estimated that a substantial percentage of first admissions with psychosis diagnosed as BD, are later re-diagnosed as schizophrenia (SCZ) and vice versa [2]. Given the heterogeneity of symptoms and overlapping symptomology some consider the current nosological categorization of mental disorders artificial, old fashioned and obsolete and alternative approaches have been proposed, like the floating continuum model, where mental disorders could be considered as gradients of pathology and not as discrete categories [3-5].

3.3 DIAGNOSIS AND MEDICATION

The hallmark clinical symptom of mania or hypomania is pressured speech, a fast and loud monologue, where topics revolve around grandiose ideas, often shifting topics. Modern diagnosis of mental disorders is currently being defined by the influential American Psychiatric Association and the Diagnostic and Statistical Manual of Mental Disorders (DSM), fourth edition [6] and the International Classification of Diseases, tenth edition, published by the World Health Organization (ICD-10) [7]. In DSM-IV, BD is classified under “mood disorders” together with major depressive disorder and the milder forms cyclothymic and dysthymic disorders. The diagnostic criteria are subject to constant controversy and revisions. DSM-V has recently been released (May 2013) and have been criticized by the lack of transparency and inventing new disorders, which some consider normal behaviour,

like for instance skin picking, binge eating and internet addiction [8], emphasising the relativistic nature and cultural context of acceptable behaviour. Further, the current leader of the National Institute of Mental Health in the US characterized DSM-V as “at best a dictionary”, criticizing its scientific validity [9]. Although controversial, there is little doubt that DSM-V remain clinically useful in a wide range of clinical settings [10].

BD I require at least one manic episode or mixed episode. According to DSM-IV, mania is defined as a period of abnormal and persistently elevated, expansive or irritable mood lasting one week or more. More specifically, the criteria for mania are: a) grandiose thoughts or inflated self-esteem b) decreased need for sleep c) increased talkativeness d) racing thoughts or flight of ideas e) distractibility f) increase in goal-directed activity or psychomotor agitation g) increase in risky behaviour. Mixed episodes are the simultaneous occurrence of manic and major depressed symptoms for at least one week. BD II is defined as a history of one or more episodes of hypomanic and major depressed episodes. The definition of mania and hypomania episodes distinguishes BD I and BD II, where hypomania is considered a milder form of mania without psychotic features.

Diagnosis require that the symptoms are not caused by other medical conditions or substance abuse, that the severity of symptoms cause social and occupational impairment, hospitalization and the exclusion of milder forms, related to BD, like cyclothymia or depression [6]. Individuals with BD I may experience psychotic episodes, especially in the manic state, while this is rarer in individuals with BD II.

The pharmacological treatment of the symptoms of BD relies on the serendipitously discovered effects of drugs in the late 40ties and early 50ties. Typical classes of drugs used to treat extreme moods in BD include lithium, antipsychotics, antidepressants, anticonvulsants and benzodiazepines, and administration of these drugs depend on the current state of the disorder and the individual response to the treatment. Example of typical treatment in BD: treatment for mania with lithium, divalproex or atypical antipsychotics. Bipolar depression treated with quetiapine, olanzapine or lamotrigine and continuation of effective mood stabilizing drugs to maintain normal mood range, and finally psychoeducation to prevent relapse and improve treatment adherence [11]. Adverse effects are common and include weight

gain, restlessness, extrapyramidal side-effects like tremors and muscle rigidity, sedation and sexual dysfunction [12]. The individual responses to these drugs tend to be relatively variable, as seen in lithium treatment in BD I where 80% have some kind of positive response [13], and 30% responds very well [14].

3.4 PREVALENCE, OUTCOME AND MORTALITY

The lifetime prevalence BD I and BD II has been estimated to be 1% and 1.1%, respectively [15]. The age of onset has shown to be heritable with three distributions with means 16, 26 and 35 [16]. The outcome of BD tends to be somewhat ambiguous as “outcome” can be defined as a full recovery and loss of symptoms or recovery of social and/or occupational function with the persistence of symptoms. DSM-IV defines partial or full recovery as two or less episodes of manic, hypomanic or depressed state over 8 weeks [17]. The course of outcome is characterized as being heterogeneous, but several factors are associated with functioning where age of onset [18], severity of symptoms [19], psychosis [20] and substance abuse [21] are all predictors of psychosocial dysfunction. Even with treatment, 37% experience relapses of mania or depression within a year [22], and 42% experience relapses within two years [17]. The yearly cost of BD in the US was estimated to be 151 billion dollars in 2009 [23] (1057 milliarder NOK). The debilitating nature of BD has a large impact on adolescent development in terms of education, social function, occupational prospects and life expectancy [24, 25]. Individuals with BD have reduced life expectancy, caused by both un-natural deaths (suicide, accidents) and natural deaths caused by unhealthy lifestyles [26]. The standard mortality rates for suicide and natural deaths in individuals diagnosed with BD have been estimated to be approximately 20 and 2, respectively [27, 28].

Although the previous facts may seem somewhat bleak, the heterogeneity in psychosocial functioning in individuals with BD should be stressed, where some may achieve historical landmarks in human achievement while other may find it difficult to accomplish daily tasks . A Swedish register study found high performing pupils in public schools had a forth fold increased risk of being later diagnosed with BD [29].

3.5 ETIOLOGY

What causes individuals to experience extreme mood swings like mania and major depressed episodes in a cyclic pattern, often with psychosis, are almost completely

unknown. Known biological, social and psychological factors which increase risk have been united in an overall bio-psycho-social paradigm, where several hypothesis have been proposed including the popular Kindling hypothesis of mood disorders [30], where life stress lower tolerance for mood symptoms in organically predisposed individuals. The behavioural approach system dysregulation model [31], where functional neural dysconnectivity results in abnormal regulation of motivation, reward and goal seeking behaviour and finally the circadian and social rhythm theory [32], suggesting a dysfunctional biological clock or life changing events that perturb accustomed daily routines.

The potency of psychoactive or antipsychotic drugs to manifest or alleviate symptoms and mood stabilizers to normalize extreme moods in particular mania, otherwise characteristic to BD and psychosis, early suggested abnormal neurotransmission during episodes and persistence of symptoms in affected individuals. A commonly held view was that fluctuating levels of the monoamines (noradrenaline, serotonin and dopamine) were responsible for the cyclic nature of BD, where high levels caused mania and psychosis and low levels resulted in depression. This is also known as the monoamine hypothesis of depression first proposed in 1965 [33]. Also dopamine has been suggested to be central in the etiology of BD, suggested by the ability to induce or alleviate mania or major depressed episodes [34], as well as glutamate [35] and GABA [36]. This rather simplified view of a “chemical imbalance” of neurotransmitters of mental disorders during the last 50 years, has served as a fruitful model in developing pharmacological drugs and understanding neural signalling [37]. Countless studies have suggested the involvement of most neurotransmitters in BD [38], an effort which is hampered by the inaccessibility of obtaining living human brain tissue and obvious ethical considerations. Clear-cut evidence of such a “chemical imbalance” in BD or any other mental disorder, is however missing. The clinical effects of the small ion, lithium as the main mood stabilizer in bipolar and the association of voltage gated calcium CACNA1C suggests channelopathy, similarly as epilepsy, may be involved in the pathogenesis of BD [39]. Anti-convulsants, the main medication in prevention of epileptic seizures, are commonly used to treat mania as an alternative to lithium, further suggesting BD is to some extent a channelopathic disorder [40].

There are numerous studies describing characteristic neural features using structural and functional brain imaging of BD patients, and the literature is substantial, but heterogeneous. Briefly, reductions in several global brain structures have been found, including reductions in total brain volume, whole brain matter and frontal lobe volume and cortical thinning [41]. A meta-study found similar changes in reduction of whole brain volume and frontal lobe volumes. This particular study suggested that age and duration of illness are key factors in these alterations, and questions if these alterations are diagnosis specific [42]. In addition, there is some controversy in determining if alterations in structural features are caused by medications or lifestyle of the affected individuals [43-45], analogously to the chicken or egg dilemma. Further, the amount of significant findings in brain alterations related to mental disorders has been subject to criticism [46], and some argue that neurosciences in general tend to be statistically underpowered [47].

BD runs in families. Although life stressors are known to increase the risk for developing BD, such as physical abuse in childhood [48] and illegal substance abuse is associated with age of onset [49], a substantial genetic contribution is revealed by several twin studies and the heritability of BD has been estimated as high as 90% [50-52]. More recently, a population based study in Sweden based on 2 million records, having a first degree relative with BD increases relative risk by ~7, and adopted children with a biological parent diagnosed with BD have 4 times increased relative risk for developing BD. The heritability of BD was in this study estimated to be 0.6 (for comparative purposes: celiac disease 0.7 [53], obesity 0.7 [54], BMI = 0.5 [55], blood pressure = 0.5 [56] and type II diabetes 0.4 [57]). The same study also found a considerable overlap with schizophrenia [58]. Although the population based heritability estimate is reduced compared to the previous twin studies, the estimate is high, and encourages genetic studies such as Genome-Wide Association Studies (GWAS).

3.6 HUMAN GENETIC AND PHENOTYPIC VARIATION

Modern human phenotypic diversity is the result of layers of past demographic and evolutionary events acting over several timescales like population expansion, migration, colonization, random genetic drift and evolutionary adaptation to diet, infections and climate. Together with inherited genetic history, the current

environment shapes contemporary traits in human individuals, like height, pigmentation or cognitive abilities. In this context, suffering from a disease or disorder can be considered a trait and there is substantial interest in identifying underlying genetic factors determining liability to complex diseases and biological knowledge which may advance understanding, treatment and potential drug discovery in treating complex diseases [59].

3.6.1 VARIATION IN HUMAN GENETICS

The inheritance of traits was first described by the well-known monk Georg Johann Mendel, experimenting with pea traits, allowing him to deduce patterns of inheritance and formulated two breakthroughs in biology: a) traits are paired, but is segregated into one copy in gametes, pairing up in the offspring b) traits are inherited independently of each other. This model was re-discovered in 1900, and more sophisticated models were proposed as the Boveri–Sutton chromosome theory in 1915 and the ideas of Nikolai Koltsov, hypothesizing a giant hereditary molecule, composed of two mirror strands, in 1928 [60]. However, it was not until 1952, that DNA was confirmed as the substrate of inheritance, identifying DNA as the genetic material of the T2 phage, followed by the discovery of the three- dimensional structure of DNA being described one year later by the combined work of Watson, Crick, Wilkins and Franklin. Watson and Crick presented in 1957 the central dogma in molecular biology, the irreversible directionality in genetics: “DNA makes RNA makes protein”, and re-stated it in 1970 [61].

Variations in human genetics have been known, through microscopy, for several decades and include chromosome copy number variation [62], rearrangements [63] and fragile sites [64]. Large scale re-arrangements, visible through microscopy by chromosome banding, usually result in a genetic condition, a consequence of altering the gene dosage of hundreds of genes. However, with increasing genetic resolution, more and more genetic variants could be identified [65], where the majority of alterations did not seem to have any observable effect [66]. The DNA sequence of the human genome was published in a draft sequence in 2001 [67, 68] followed by re-vision and completion in 2004 [69], and brought with it several fundamental realizations: a) the human genome was composed of approximately 21,000 genes, far below most estimates b) the majority of the human genome is seemingly non-coding and non-functional c) humans are strikingly similar to each

other with only ~0.1% differences in DNA sequence. Although humans are highly similar to each other, due to recent bottleneck events during speciation, genetic differences can easily be detected. Re-sequencing efforts have showed a larger genetic difference that previously though, where sister chromatids, within the same person, are 99.5% identical [70]. This non-negligible common genetic variation between human individuals is thought to influence phenotypic variability including susceptibility to complex diseases like cancer, hypertension and mental disorders.

Genetic differences between humans can be divided into the following categories: single nucleotide polymorphisms (SNPs), small insertions and deletions (indels), copy number variants (CNV) and structural variations. The Human Genome Project [67], the SNP consortium [71] and the International HapMap Consortium [72] have collectively identified approximately 10 million common SNPs in a limited amount of samples. This figure has been recently adjusted by the 1000 Genomes Project which included 1092 genomes from 14 world-wide populations and identified 36.6 million SNPs, 1.38 million small indels and 13,800 large deletions. Individuals, on average, carry 3.6 million SNPs, 350,000 small indels and ~700 large deletions [73].

The most abundant category is SNPs, or ancient point mutations. Point mutations are substitutions of a single base pair in DNA, compared to another genome. Point mutations spontaneously form, due to the near-perfect molecular mechanism of DNA replication during cell division and formation of the gametes. The average mutation rate pr. DNA base pair is estimated to be ~1.1×10^{-8} pr. site in the haploid genome [74]. Having 3,101,804,739 (Ensembl GRCh37.p12, Feb 2009) base pairs in the human genome, gives 34 novel mutations per generation (x 2 for sister chromatid). The fate of these point mutations are subject to evolutionary forces, and if a point mutation is distributed within a population at a frequency above 1%, that mutation is denominated a single-nucleotide polymorphism (SNP) [75]. The frequency of any given SNP within a population, generally, reflects the age of the point mutation event. For example, SNPs shared across all human populations originates before “Out of Africa” migration, although they might have very different frequencies between populations [76]. The overwhelming majority of SNPs have no functional consequences on protein coding DNA sequence, where 0.01% is nonsense SNPs, 0.02% are frame shift SNPs and 0.8% are missense SNPs (dbSNP 139).

Despite the fact that genetic linkage studies were wildly successful in identifying the genetic determinants in diseases having a Mendelian pattern of inheritance, also known as monogenic diseases, the methodology failed to identify causal genes when applied to common diseases and disorders like most cancer types, infertility, obesity, auto-immune diseases and mental disorders, conditions which show substantial degrees of being heritable. It became apparent, that these diseases were not monogenic traits, and the failure to identify clear susceptibility genes, suggested a complex inheritance and the need for a different approach altogether [77, 78]. Two main hypotheses were proposed in 2001 to explain the nature of genetic susceptibility of complex diseases: common disease – common variant model [79] and the common disease – rare variant model [80], also known as the CDCV vs. CDRV debate [81]. The CDCV model got a head start after the emergence of efficient genotyping technologies based on microarrays able to measure the allelic state of hundreds-of-thousand SNPs, and the formation of the international HapMap consortium [75], a project aimed at producing reference datasets of common SNPs in several populations. This resulted in a massive effort striving to identify SNPs conferring susceptibility to complex diseases in large case-control studies, including the hallmark Welcome Trust Case Control Consortium study [82]. For the first time, SNPs possibly involved in forming susceptibility to complex diseases, diseases which affect a substantial proportion of a population and are costly for society as a whole, could be identified, promising to provide molecular insights of associated genes [83].

3.6.2 VARIATION IN HUMAN PHENOTYPES AND HERITABILITY

All humans are phenotypically unique, the net result of a unique genetic inheritance and how genes interact with the current environment. Even monozygotic twins obtain genetic alterations over time, which may result in phenotypic differences and susceptibility to disease [84]. A phenotype is the observational set of one or more traits, and as genes tend to control the observational set of traits, phenotypes are more or less inherited. The question is to what extent, or the degree of inheritance. The influence of genes on a trait is in genetics coined “heritability”. Heritability is often misinterpreted as the degree of inheritance or “chance to catch the trait” in an offspring or proportion of a trait that is genetic. Heritability is a technical term in genetics and is defined as the proportion of phenotypic variation that is due to

genetic variation in a specific population [85]. A heritability of 0 equals no genetic contribution to phenotypic variation and a heritability of 1 equals all phenotypic variation are caused by genetic variation. As simplified examples, the heritability of black hair in Asia or being born with ten fingers is 0 (no phenotypic variation) and all monogenic disease have a heritability of 1 (no genotypic variation). The heritability of a complex trait is not constant and can shift with changes in environment and/or genetic constraints imposed by for instance natural or artificial selection [85]. Elucidating the genetic basis of complex diseases, the heritability measure can be informative, as one can define the trait of interest and measure the genetic variation, but not the environment. Thus, heritability estimates can indicate the feasibility of genetic studies of complex disease including BD.

Most human traits are the result of genetic drift, random genetic changes which happen to manifest traits, like hitchhikers thumb or dry earwax, while other traits have been subject to natural selection like skin pigmentation or lactose tolerance [86, 87]. In modern genetics the phenotype is not the classical set of observational traits like wrinkles on peas or colour in butterfly wings, but used on disease states or conditions which may not be observational per se, like hypertension or mental disorders. These phenotypes have been considered, by some, as too broad and the need for more narrow phenotypes, at a more intermediate level, like blood lipids or brain activity has been advocated. Such unobservable or internal phenotypes have been coined “endophenotypes” [88].

3.6.3 ENDOPHENOTYPES

In genetic epidemiology a good definition of the phenotypic traits of interest, is desirable in order to avoid clinical heterogeneity within the trait of interest [89]. There has been substantial discussion about the usefulness of psychiatric diagnosis and if they serve as good phenotypic traits [88, 90]. The rationale behind this discussion is that mental disorders can be considered as somewhat crude and heterogeneous categories and behavioural symptoms as high level phenotypes, results of possible malfunctioning low level biological processes, such dysfunctional neural activity. By identifying measureable and heritable components at a more intermediate level, between the disease and genotype levels, would possibly strengthen the ability, by larger effect sizes, to detect genetic findings [88, 91, 92]. In the case of the high level phenotypes schizophrenia (SCZ) and BD, working memory and response to

emotionally charged faces have been, respectively, suggested as possible intermediate endophenotypes [93, 94]. As a result, functional MRI (fMRI) experiments measuring the neural activation patterns during these tasks can be used as endophenotypes, which may reveal possible characteristic neural patterns of these disorders [92].

3.6.4 FUNCTIONAL MRI AS ENDOPHENOTYPES

The human brain consumes large amounts of energy. During controlled stimulation, for instance by solving a cognitive task, activated brain areas have elevated neurotransmission, resulting in increased metabolism, demand for oxygen and increased blood flow to that area. The Blood-Oxygen-Level Dependant contrast, or BOLD response, discovered by Seiji Ogawa in 1990, exploits the magnetic properties of haemoglobin, which is paramagnetic when deoxygenized, allowing in vivo detection of real-time activation patterns [95], permitting identification of task related areas in the brain. Several aspects of brain structure as well as several cognitive abilities are seemingly under biological control [92, 96-99]. Combining genetic data with the endophenotypes of brain imaging, may allow detection of SNPs which influence brain activation patterns, measured by functional MRI [92]. Several SNPs and associated genes have been proposed to influence activation patterns including the schizophrenia susceptibility genes COMT [100] and ZNF804A [101], although these and most other studies do make biased assumptions of regions of interest or select a candidate gene of interest. Defining a priori regions of interest or candidate genes is generally not recommended since the current understanding of gene function on brain regions remains limited. Hypothesis free, whole genome and whole brain studies do not make a priori assumptions and promises to deliver a global genetic and neurological context, also known as voxel-wide GWAS [102].

Combining and statistically testing genome-wide data with whole brain images, using univariate statistics, results in a large number of statistical tests and the approach suffers under a unresolved multiple testing problem [103, 104]. Both data types contain a substantial degree of correlated data points and redundancy, and data driven techniques which construct new and uncorrelated measurements can be used in voxel-wide GWAS, including Principal Component Analysis (PCA) [105] (paper II) and Independent Component Analysis (ICA) of fMRI data [106].

3.7 GENOME-WIDE ASSOCIATION STUDIES

It was not until the beginnings of 1990ties that the genetic determinants of medical conditions with Mendelian inheritance patterns could be identified using genetic linkage, as seen with the identification of cystic fibrous gene in 1989 [107]. Linkage studies take advantage of recombination events between sister chromatids [108]. If most recombination events in a related family are known, as well as the medical status of each individual, inherited chromosomal regions between the affected individuals and thus shared genetic regions harbouring a dysfunctional gene, can be identified and potentially verified through DNA sequencing [109]. This approach was first used on candidate regions, but later whole genome-wide analysis became technically possible [110]. Genetic linkage studies rely on measuring genetic elements that tend to vary between individuals, thus tracing the pattern of inheritance between related individuals. Several types of genetic variation can be measured to trace the inheritance of stretches of DNA including point mutations (SNPs) and tandem repeats (microsatellites). Microsatellite markers were the most common tool to trace the genetic inheritance in linkage studies [111]. Microsatellite markers was later replaced by single SNP markers [112], as SNPs are more common and have a more dense and uniform distribution on the genome, allowing an increase in genomic resolution and capability to detect very short stretches of DNA conferring disease susceptibility. Studies measuring SNPs in a population and statistical testing them for association to a trait of interest, is known as genome-wide association studies (GWAS).

The GWAS methodology has its origin from traditional gene expression studies, where thousands of oligonucleotides, representing genes, are printed on a glass slide, taking advantage of the extreme specificity of DNA hybridization [113]. Following fragmentation and probe hybridization of a reversed transcribed mRNA samples (cDNA), global gene expression can be measured, by luminescence, followed by scanning and digitalization. After digitalization, normalization and quality control, statistical testing follows, a handful of genes may be found significant, and invites biological interpretation [114]. The scale and number of genes to interpret gradually increased, as did the difficulty in interpreting the results [115]. Analogously to global gene expression studies, GWAS, measuring the qualitative allelic state of SNPs in a panel of individuals, are characterized as hypothesis free experiments,

overcoming biased prior knowledge and incomplete understanding in disease pathophysiology [83]. A landmark GWAS was the Welcome Trust Case-Control Consortium (WTCCC) in 2007, reporting findings in seven complex disorders [82]. This study sparked numerous follow-up studies and new initiatives aiming to further disentangle the genetic susceptibility to complex disorders, including mental disorders [116], promising to deliver molecular insights in complex disease pathology and the possibility of identifying novel drug targets. Experimentally measuring SNPs, also known as genotyping, rely on a large but limited amount of highly informative SNPs.

3.7.1 LINKAGE DISEQUILIBRIUM

Genetic linkage is a phenomenon in biological inheritance, and linkage disequilibrium is a statistical measurement for genetic linkage [117]. Genetic linkage is the tendency for neighbouring stretches of DNA to be inherited together, and complete linkage is the absence of recombination within a population. The degree of linkage can be measured, by linkage disequilibrium (LD), and can be used to intelligently select a limited amount of SNPs which capture the majority of genetic variation or to infer missing data. For instance, even though 38 million SNPs are known in human populations, only a fraction of the most informative SNPs needs to be measured in order to conduct genome-wide studies. LD is mainly measured by the squared Pearson's correlation coefficient or r^2 between two SNPs, and can range from null to one. A pairwise r^2 value of 1, between two SNPs, is interpreted as if the two SNPs are in complete linkage and the two markers provide the same information, and therefore one is redundant. LD is useful when selecting SNPs for genotyping platforms, where the most genetically informative SNPs are selected [118]. The HapMap consortium has provided reference data for calculating LD in various populations [75]. The most informative SNPs are known as tag-SNPs, and by typing ~500,000 carefully selected SNPs, 80% of total human genetic variation in a population are covered [119], and even less for familial linkage studies.

3.7.2 QUALITY CONTROL

GWAS are powerful tools, but several issues must be addressed in order to avoid spurious findings [114]. Quality control (QC) of the called SNP states is essential, as several confounding factors may cause artificial findings. Some of these factors include missing data, duplicate samples, fulfilment of Hardy-Weinberg equilibrium,

conflict in predicted and annotated gender, cryptic relatedness, inbreeding and population stratification [120]. Also the general volatility and sensitivity of the method to changes in experimental conditions and equipment should be stressed, and could lead to confounding caused by, differing DNA extraction protocols, batches of cases only, shipping conditions or choice of technological platform [121]. Even though significant findings may be found, additional testing like replication in independent samples, fine mapping and re-sequencing are required to identify a causal variant [89]. As an example, a GWAS reporting significant SNPs associated with longevity, was later found to contain artefacts and unexpected “behaviour” of a subset of SNPs, resulting in retraction of the article [122].

3.7.3 IMPUTATION

Genome-wide SNP genotyping, measures the allelic state of tag-SNPs, a subset of all known SNPs. As the SNP genotyping technology developed, different commercial platforms used different strategies for selecting tag-SNPs, complicating replication studies and collaborative efforts. The two competing companies Illumina and Affymetrix, used different chemistry and strategies in selecting tag-SNPs, which resulted in a low direct overlap of selected tag-SNPs [123]. However, tag-SNPs can be used to computationally and statistically infer the state of un-genotyped SNPs, using the LD structure of a reference population, a method known as SNP imputation [124]. SNP imputation is essential in collaborative efforts and mega- and meta-analyses, combining different genotyping platforms and methods for SNP imputation have been developed [125, 126]. SNP imputation results in statistical approximations of allele states, also known as SNP dosage.

3.7.4 STATISTICAL ANALYSIS OF GWAS

As SNPs are categorical, they can be summarized in contingency tables and several statistical tests can be performed using different assumptions, depending on the study design and can be divided into two main tests: genotypic or allelic test [127]. The most common test is the allelic 2x2 Pearson’s chi-squared test, which assumes an additive effect of alleles. The most common study design in GWAS is the observational case-control studies, where the Pearson’s chi-square test or a modification of this test called Cochran–Armitage trend test of the minor allele count, with one degree of freedom, are used to test for derivations in SNP frequencies

between cases and controls. Cohort studies measuring a qualitative trait of interest are also common, for instance brain activation (paper II), using logistic or linear regression models which allow additional dependant variables like sex or age into the model. The mathematics of these models are not elucidated here, see review [127].

A naïve analysis would conclude that significant findings are caused by differences in SNP frequencies between the cases and controls populations. However, in population genetics other factors may skew allele frequencies, like cryptic relatedness, population stratification or inadequate quality control. The non-random differences in allele frequencies between populations, caused by the varying ancestry of populations and to a lesser extent natural selection, are known as population stratification, and is a confounding factor in large, multicentre GWAS studies, as demonstrated by the association of SNPs in LCT and height [128]. The most common method to adjust for population stratification is to conduct a principal component analysis of the SNP data and include several of the retuning eigenvectors as independent variables in a logistic regression analysis [129]. This relatively simple method has shown to be remarkably efficient, where the genetic variants in individuals between populations re-create a near perfect map of modern day Europe [130]. Case-control studies, using logistic regression which includes the population eigenvalues as independent variables, are the most common approach in multicentre case-control studies.

Since GWAS m statistically tests hundreds-of-thousands of null hypothesis at once, correction for multiple testing is in order. The frequentist solution was the conservative Bonferroni adjustment method, and a general p-value of $p < 5 \times 10^{-8}$ was conceived as the golden standard for statistical significance in GWAS [131, 132]. Although conservative, this method and most methods for adjusting p-values for multiple testing assumes that the tests are independent, this assumption being immediately violated by LD between SNPs, leading to over-adjustment and an overall increase in type II errors (FN). Although overly conservative, it guaranties the absence of type I errors (FP) and it is assumed that clinically important SNPs with large effects surpass this threshold. Hundreds of GWAS studies have reported numerous significant findings in complex diseases as listed in the NIH GWAS catalogue [133].

For instance the WTCCC study included both the Cochran–Armitage trend test and genotypic test, as well as an empirical Bayesian method [82]. In the case of the first PGC SCZ GWAS, logistic regression of imputed SNP dosages using three principal components of population structure as covariates, was used in the stage 1 mega-analysis, and results revolved around speculation of single gene functions and no reported systematic efforts, like gene set analysis, was made to interpret the findings [134]. With some exceptions, the majority of all GWAS returned astonishingly few SNPs with large effects were the mean OR of all significantly associated SNPs is 1.33 [133].

3.8 INTERPRETATION OF GWAS

The first generation of large GWAS reported significant SNPs associated with a trait or disease in tables and a Manhattan plot, a genome-wide plot of SNP significance across chromosomes, but little effort was spend on systematic analysis of biocurated information of associated genes [82], possibly due to the limited amount of SNPs passing the stringent threshold of significance. Prior to GWAS, global gene expression studies encountered the same challenge of biologically interpreting hundreds of significant differentially expressed genes, associated with an experimental condition. This was a driving force in the development of bioinformatical tools aiming to detect biological similarities within a group of genes, based on biomedical knowledge about genes. Biomedical knowledge about genes is systematically annotated across numerous databases [135]. The sources of such biomedical information may be diverse, ranging from shared biological context as indicated by shared Gene Ontology terms [136], recurring words in PubMed abstracts or shared functional domains within the protein sequence. Bioinformatical tools for performing such analysis are abundant and among the first and most successful include DAVID [137], and GSEA [138]. Although highly similar to gene expression studies, the GWAS methodology has its own unique issues when interpreting results, as they produce a list of SNPs, and not a list of genes.

Biomedical knowledge about the genome almost entirely revolves around protein encoding genes, represented by their physical positions at the start and stop codons. As genes occupy approximately 2% of the genome, it is not surprising that the majority of SNPs are located outside the physical boundaries of genes [139]. It is

common practice that biological interest declines with increasing physical distance towards genes. This is simplistic. Take lactase persistence, the autosomal dominant trait to digest lactose, as an example. Here, SNPs within two enhancers, determine the expression of the LCT gene in adolescence and adulthood. The enhancers are located 14 kbps and 22 kbps upstream of the LCT gene and is physically located within introns of the neighbouring MCM6 gene [140]. A naive and hypothetical GWAS of lactase persistence would find the SNPs within MCM6 highly significant, however the role of MCM6 (a DNA helicase) in lactase persistence, would be somewhat puzzling. This is a simple example of one of the most important challenges when interpreting GWAS results: assigning SNPs to genes.

3.8.1 ASSIGNING SNPS TO GENES

In conventional GWAS, each SNP is tested for association to a phenotype of interest also known as the single-marker approach [114], and results are presented as a ranked list of significant SNPs, where further analysis and interpretation are based upon biomedical knowledge of nearby genes [141]. The most simple and common method to annotate SNPs to genes, is based by physical distance, where a SNP of interest is assigned to the closest gene or genes within a specified distance to the SNP. Specifying such a distance is somewhat arbitrary but ten, twenty, fifty or hundred kilo-base pairs (kbps) up- or downstream of the SNP is often used. Studies combining SNPs with gene expression, aiming to detect cis-regulatory SNPs which affect gene expression, also called eQTL studies, have found that the majority of SNPs affecting gene expression tend to be located within 20 kbps of the gene it exerts its influence [142] (less than 5% are located more than 20kb away from gene). However, long range enhances are well known to be involved in monogenic conditions where for example regulatory elements 125 kbps downstream of the PAX6 gene cause aniridia, X-linked deafness type 3 and deletion of non-coding sequence ~1000 kbps upstream of POU3F4, and lastly polydactyly and the long range ~1000 kbps upstream enhances of the gene SHH [143]. In the case of lactase persistence the two influential enhancers are located 14 and 22 kbps upstream and within the MCM6 genes. For instance, a SNP located within a gene and 20 kbps upstream of another gene, the first annotation may be kept for further analysis, and the second ignored. In the above example, SNPs within the lactase (LCT) enhances, would then be annotated to the MCM6 gene, missing LCT altogether. However, the

enhancers influencing LCT expression are within 20 kbps and are also in high linkage-disequilibrium with SNPs within the LCT gene. Taking advantage of both distance and LD between SNPs to aid SNP-gene annotation, have increasingly been applied in assigning SNPs to genes including the developed methods and software “LDspline” (not available) and “ProxyGeneLD” (Perl script) [144, 145], with the added benefit of inferring un-genotyped SNPs to genotyped SNPs using a HapMap population reference panel. This also includes “LDsnpR” in paper I.

Physical position is most often used in assigning SNP to genes, and the significance of individual genes most often involves taking the minimum p-value of SNPs within the gene to summarize the gene overall significance to the trait of interest. As a consequence gene length is an important factor to consider, as larger genes contain more SNPs and, by chance, are more likely to contain significant SNPs. The median gene length is 20kbps with a standard deviation of 121 kbps, reflecting a high degree of variability of the genomic architecture of genes. This is a particular problem in neurological genetic studies as genes involved in the nervous system tend to be longer, thus neurological genetic studies are prone to find neurological related genes by chance [146, 147]. In addition, differences in LD structure and gene clustering on the genome have been also shown to be confounding factors [147]. Other methods for aggregating SNPs to single gene statistics can be to Bonferroni adjust the minimum p-value within the gene or the SNP ratio test, using the second best p-value or to compare the ratio of significant SNPs (typically $p<0.05$) versus all SNPs within the gene [148]. The variation in gene length, non-random localization of genes on the genome is important to keep in mind when assigning SNP to genes, especially since paralogous genes, gene duplications and subsequent functional divergence, tend to be physical neighbours, but is non-trivial to adjust for these confounding factors [144], see figure 1.

3.8.2 GENES TO GENE SETS

Gene sets are usually pre-defined and can be derived from high-throughput experiments, computationally predicted or manually annotated using literature, and can be of varying quality. One of the first initiatives in systematic biocuration was the Gene Ontology (GO) consortium, an effort to annotate biological meaning to genes using a controlled vocabulary [136], and is still commonly used in gene set enrichment studies. GO terms are organized into three different knowledge domains

about genes: biological process, molecular function and cellular component, where the terms within the three domains are organized in a hierarchical structure starting with genetic terms and, going down the hierarchy, increasing the terms specificity. Genes are usually annotated with one or more terms from the aforementioned domains. These GO terms can be used to test if a group of genes share any terms, possibly reflecting a shared biological context of the genes of interest. This approach is common and during the last decade, and has routinely been used to interpret results from high throughput experiments. Other sources include metabolic pathways KEGG and Panther [149, 150], as well as sequence features Pfam [151], protein-protein interacting networks [152] or text mining in PubMed abstracts [153].

3.8.3 STATISTICAL GENE SET ANALYSIS

Several methods to test the statistical enrichment of biocurated information within a gene list have been developed. The methods have their origin in analysis of gene expression studies, and can be divided into two main categories: self-contained (association) or competitive (enrichment) tests, where the first test “no gene sets are associated with the phenotype” and the latter tests “none gene sets genes are no more associated with the trait than the non-gene set genes”. Although these two hypotheses seems very similar, a distinction is often made [141, 154], although the competitive test are wildly more popular. Typical testing of the competitive hypothesis includes contingency tables with a following hypergeometric test or the GSEA algorithm, see figure 1.

The most common scenario is to have a list of significant genes from a GWAS, and testing the competitive null hypothesis that no gene sets contain more significant genes then non-gene set genes. The most frequently used method is to construct 2x2 contingency table for each gene set, and calculate a p-value based on a discrete, univariate probability distribution, most often the hypergeometric distribution [155]. Also the Fisher’s exact test and the binomial z-tests are used, and these methods differ little in the assumptions being made and produce similar results. However, the 2x2 contingency tables approach also called over-representation analysis, has been criticized for being dependant on the initial threshold of statistical significance, being sensitive to the reference gene background and for not taking into account interdependency between gene sets [156, 157]. Ranked based methods leverages the degree of significance of all genes and a modified version of the Kolmogorov-

Smirnov test, called the GSEA algorithm, has been wildly successful in gene set analysis of gene expression studies [138]. However, the GSEA algorithm requires a summarized value like fold expression or significance for all genes, and may be non-trivial to ascertain for GWAS results. This method has been criticized to be too biased towards the best scoring genes. Both over-representation analysis and the GSEA methods are well established, but there is no gold standard for gene set analysis and numerous tools have been developed [158, 159]. In this thesis we make use of over-representation analysis (paper II and III).

The first gene set analysis of a GWAS was a study using the GSEA algorithm of GWAS of Parkinson's disease, reporting two significant gene sets [160]. One year later a gene set analysis of all seven WTCCC phenotypes using the hypergeometric test of contingency tables was used, suggesting multiple biological plausible findings, albeit ambiguous statistical significance [161].

There are some considerations with gene set enrichment methods caused by confounding factors, see figure 1, and these considerations are best handled by sample permutation based methods [162]. Permutation based methods (producing null results by sample randomization) is, by many, considered the golden standard, as it accounts for confounding factors like linkage disequilibrium, gene length and SNP coverage present in a GWAS. Algorithms for sample permutations include GSEA-SNP [163], GSA-SNP [164], GENGEN [160] and SNP-ratio test [148]. However, sample permutation requires the availability of raw genotypes, and as most GWAS are large multicentre mega- or meta-studies, the raw genotypes can be difficult of obtain and are not publically available, a consequence of the controversy in the identifiability of genomic data [165]. Also, there is a considerable computational cost. For instance performing 100,000 permutations of a large GWAS by using standard GWAS software, PLINK, and would take years to complete [162]. Methods which does not require raw genotypes and uses a SNP list with p-values include ALLIGATOR [166], MAGENTA [147] and i-GSEA4GWAS [167], although these methods are prone to the aforementioned confounding factors. One approach, which does not require sample randomization, is to create aggregate gene scores, which aims at taking into account the variable degree of number of SNPs within a gene [147]. In this thesis, sample permutation test (paper II) and Fisher's exact test (paper III) is used to assess the significance of gene sets.

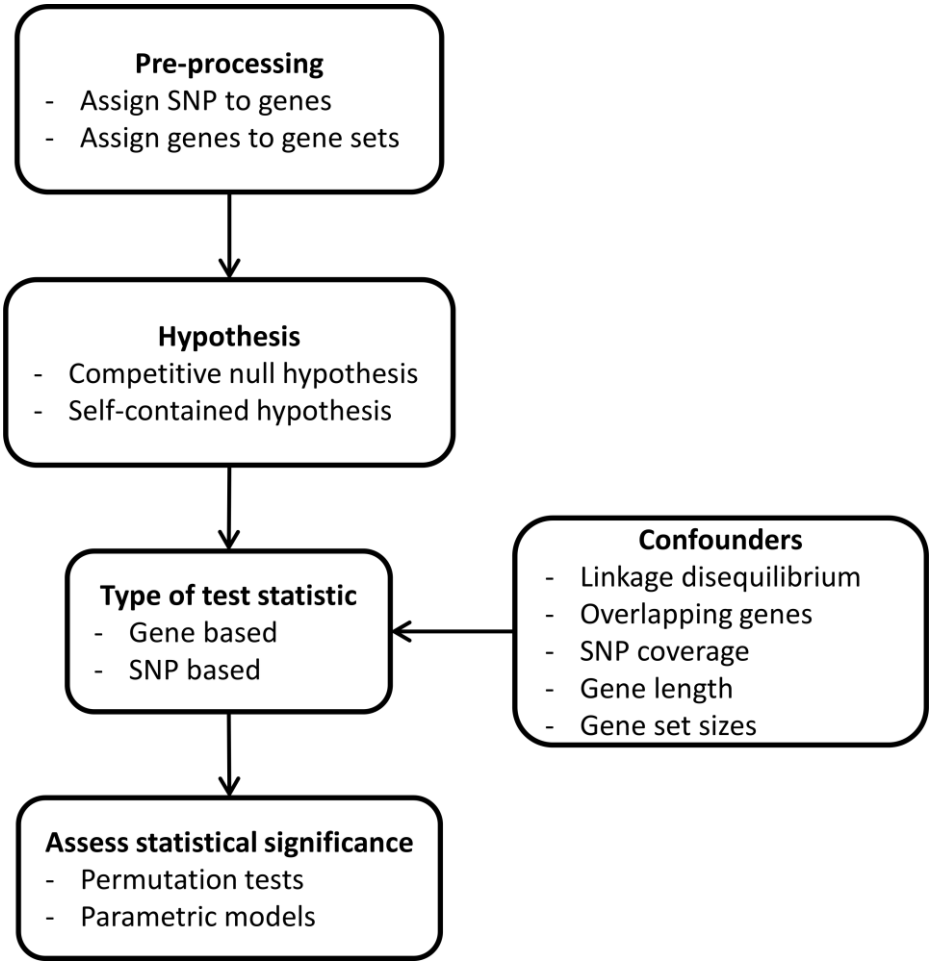

Figure 1: Essential steps for assessing the significance of gene sets from SNPs associated with a trait or disease. Adopted from [159].

3.8.4 GENETIC FINDINGS IN GWAS OF BIPOLAR DISORDER (2010)

Several genomic regions have been implicated using linkage studies [168], but without converging findings. The development of effective SNP genotyping methods, GWAS studies allow for far larger samples, able to statistically detect the weak effect sizes of single SNPs. The WTCCC study presented the first comprehensive GWAS of BD, which included 1868 cases and 2938 controls, reporting four significant findings ($p<5 \times 10^{-7}$), where the closest genes were KCNC2, GABRBI, GRM7 and SYN3 all related to membrane transport of neurotransmitters and synaptic function [168]. Subsequent GWAS of BD included (cases = 1461 and 2008 controls), found no significant findings [169] while another study reported one significant finding, ANK3 (cases 1098 and 1262 controls) [39]. Further increasing samples in the studies (cases=2076 and 1676 controls), reported no significant findings, where the most associated region was 1.8×10^{-7} and ITIH1 [170]. As the GWAS field

developed including imputation methods increasing the number of SNP tested, a stricter statistical significance was as conceived as a new gold standard (nominal p value < 5 x 10^{-8}), with further requirement of replication of findings in independent samples [171]. The difficulty in replicating findings and missing statistical significance, lead to the formation of the Psychiatric GWAS Consortium aiming to unite efforts in GWAS in psychiatric genetics [116]. Although the aforementioned studies reported significant findings, no clear cut susceptibility genes with large effects was found.

3.8.5 GENE SET ANALYSIS IN BIPOLAR DISORDER (2010)

Several studies have reported findings using gene set analysis of GWAS and SNPs associated with BD. Here four gene set enrichment studies of the WTCCC BD GWAS is summarized: In one of the studies the authors developed their own algorithm (ALLIGATOR) [166] and tested for enrichment of GO terms in SNPs associated with BD in a meta-analysis [39]. In the study they used different p-value cut-offs and identified several broad categories including “autophagy”, “hormone activity” and “RNA splicing”. Another study “found heparan sulfate and heparin metabolism”, “cytoskeleton remodelling”, “niacin-HDL metabolism” and “glutamate regulation of dopamine D1A receptor signalling” significant, using the top 2.5% associated genes and assessed significance by comparing the bottom 2.5% to the top 2.5% genes using an ad hoc scoring scheme [161]. A third study of the WTCCC BD GWAS reported “inositol metabolism”, “chondroitin sulphate biosynthesis” and “MAPK signalling pathway” as significant over-represented gene sets [172], while a fourth found “ion channel activity-related” terms significantly over-represented in the WTCCC BD GWAS [173]. These studies are difficult to compare because they all use of different methods including assigning SNP to genes, summing p-values pr. gene, different sources for gene sets and different algorithms and tool for performing the actual gene set analysis, indicating a need for a gold standard [141]. Also the aforementioned studies are limited in that the WTCCC GWAS of BD, which the gene sets analysis are based, is somewhat underpowered to identify the very small effects of SNPs, limiting the possibility to detect recurring signals between related genes [174].

4 AIMS OF THE THESIS

The overall aim was to gain a better understanding of the molecular mechanisms involved in the disease pathology of bipolar disorder (BD) by integrating biomedical information about genes, known as gene set analysis, to aid interpretation of on-going genome-wide association studies (GWAS).

The first specific aim was to improve methods for assigning SNPs to genes by using physical position together with inheritance patterns (linkage-disequilibrium), as downstream analysis of GWAS like gene set analysis, require careful SNP to gene assignment.

The second specific aim was to improve statistical power and conduct a GWAS of a functional MRI experiment. Statistical power is increased on two levels: 1) by principal component analysis of fMRI data and use the reduced data as endophenotypes in GWAS and 2) preform gene set analysis of moderately associated SNPs.

The third specific aim was to perform a gene set analysis of a GWAS of BD using protein-protein interaction data as a contextual filter. Here we use the results from a re-analysis using empirical Bayesian statistics of emerging GWAS results of BD.

5 MATERIALS AND METHODS

The three papers included here are based on different materials, but all include the genetic material from the TOP sample. In Paper I we used summary statistics from three GWAS of bipolar disorder (BD), including the BD case-control dataset of the TOP study. In paper II we used SNP data and functional MRI brain imaging data from the TOP study. In the final paper III we used summary statistics from a re-analysis from the most recent PGC BD GWAS, which include the BD and control samples from TOP.

Table 1 Summary of data type, size and samples used in this thesis.

	Study	Phenotype	Cases	Controls	Data type	Ref.
Paper I						
	WTCCC BD GWAS	Bipolar	2938	1868	summary statistics	[82]
	TOP BD GWAS	Bipolar	336	198	summary statistics	[175]
	A German BD GWAS	Bipolar	1300	682	summary statistics	[176]
Paper II						
	TOP genotypes	fMRI BOLD	138	108	SNP genotypes	[177]
	TOP fMRI	fMRI BOLD	138	108	fMRI voxels	[177]
Paper III						
	PGC2 BD cmFDR	Bipolar	15,795	22,365	summary statistics	-

5.1 THE THEMATIC RESEARCH AREA PSYCHOSIS (TOP)

The Thematically Organized Psychosis Study (TOP) is a large translational study at University of Oslo in collaboration with several psychiatric hospitals in Norway, aiming to identify biomedical factors involved in onset and persistence in psychotic disorders, with focus on the psychosis disorders bipolar disorder (BD) and schizophrenia (SCZ). Inclusion criteria include: between 18 – 65 years, speak a Scandinavian language, and fulfil the diagnostic criteria, according to DSM-IV. Exclusion criteria were defined as subjects who had suffered major brain trauma or any developmental disorders. Diagnosis was set by trained psychiatrists or specializing MDs, and reliability of diagnosis and overall agreements for the DSM-IV categories has been estimated to be 82%. Included individuals were interviewed to register a number of clinical, neurocognitive and psychosocial assessments including

onset, relapses, education, alcohol, illegal drug abuse and medication. Several neurocognitive and psychosocial functioning were assessed by the following scales YMRS [178], PANSS [179], IDS [180], GAF and WASI [181]. The healthy control subjects were randomly recruited from the same areas as the patients, and interviewed with focus on demographic and clinical information. Inclusion criteria were: born in Norway and between 18-60 years. Exclusion criteria were: suffered from head injury, mental retardation or neurological disorders or a history of mental disorder or close relatives with mental disorders indicated by the Primary Care Evaluation of Mental Disorders questionnaire [182]. The TOP project has recruited a large number of participants and has currently genotyped, (in the TOP8 data freeze) ~1577 samples passing quality control of which, 421 are healthy controls, 587 have BD (BD I, BD II and bipolar NOS) and 411 have a SCZ diagnosis (schizophrenia, schizoaffective and schizophreniform) and 158 have with other diagnosis (including major depression and other psychosis).

5.2 ETHICAL CONSIDERATIONS

All participants in the overall TOP study gave informed written consent. The TOP study has been approved as a bio-bank by the Regional Committee for Medical Research Ethics, the Norwegian Data Inspectorate, and the Norwegian Health Authority. All genetic and neuroimaging data used in this thesis were de-identified before being received, and the identification key have never been used nor been accessible. However, there have been some controversy and discussion of the traceability of anonymized genome wide data, resulting in strict data sharing and storage policies. All genetic and imaging data used in this thesis has been stored on password protected and offline external disk drives and will be deleted after the completion of this thesis.

The participants do not necessarily benefit directly from the results presented in this thesis, although method development in GWAS in general and in neuroimaging genetics may, in the long term, provide better understanding of the biological underpinnings of BD.

5.3 GENOTYPING AND QUALITY CONTROL OF THE TOP SAMPLE

Part of this thesis included calling the discrete SNP states from raw data and performing quality control of the TOP sample. The TOP sample was genotyped on

the Affymetrix Genome-Wide Human SNP Array 6.0 platform, measuring 906,600 SNPs, performed by Expression analysis Inc., Durham, USA and later, at the genotyping core facility at the Oslo University Hospital, Norway. Provided with 2235 raw data files, quality control was performed on two levels, using SNP data and using information about the individual samples. First, all raw data were read using Affymetrix Power Tools, and the birdseed algorithm version 2 within, was used to call the SNP states of the raw data. To avoid batch effects, genotype calling was performed in each batch separately (n=33). The called genotypes for all samples were then merged and transformed from AB (Affymetrix convention), to actual state (ATCG). Then all SNPs were ensured to be located in the same strand (forward strand), and genomic physical position (hg18) was provided by Affymetrix annotation files. In the next step several sanity checks were performed using PLINK by clustering based on pairwise identity by state (IBS). This resulted in the detection of sample duplicates (n=391), cryptic duplicates (n=77, same sample, different diagnosis) and siblings (n=9 removed, keeping one). Samples with a discrepancy between reported and predicted gender were removed (n=24). SNPs significantly associated with batch identity were set to missing ($p<1x10^{-8}$). Then the TOP dataset was merged with the reference HapMap III population, and a new IBS clustering was performed to identify TOP individuals with a non-European origin, which were visually identified by a cluster plot and excluded (n=75). GWAS tend to use different threshold for missing data and here a liberal threshold of maximum 5% missing data was allowed, further excluding 99 samples. After the initial quality control, 1577 samples remained, denominated as the “TOP8 QC1” dataset. Further quality control of this dataset has been recommended, including minor allele frequency, missing rate per SNP and pr. individual and fulfilment of Hardy-Weinberg equilibrium.

5.4 FUNCTIONAL MRI EXPERIMENT

Some TOP study individuals participated in several functional MRI (fMRI) experiments, including a negative faces paradigm, measuring the hemodynamic response in the brain when subjects were given a task to match negatively charged faces (sad, angry, frustrated), an experiment designed to measure amygdala activity [183]. Data collection, quality control and analysis of this fMRI experiment were performed by co-authors of paper II, and are included here for the thesis to be self-contained.

The task was to determine which of two images at the bottom of the screen, was an exact match with a target image at the top. The images displayed were either negative (angry or afraid) faces (experimental task) or geometric figures (sensorimotor control). The individuals solved the tasks with a mean response time of 1155 Ms and an accuracy of 98.59%. A block design was used that considered consisting of four experimental and five sensorimotor blocks lasting 32.64 seconds each. The sessions took 5 minutes and 10 seconds to complete including eight dummy disk acquisitions. E-prime software (Psychology Software Tools, Inc.; Pittsburgh, PA) controlled the presentations of the stimuli while using VisualSystem (NordicNeuroLab, Bergen, Norway). Responses were collected using ResponseGrips (NordicNeuroLab Bergen, Norway).

MRI scans were acquired on a 1.5T scanner (Siemens Magnetom Sonata, Siemens Medical Solutions, Erlangen, Germany) supplied with a standard head coil. 152 volumes (24 axial slices of 4 mm with a 1 mm gap between slices), covering the full extent of the brain, were acquired using an EPI BOLD sequence (TR= 2040 Ms, TE= 50 ms, flip angle=90°, matrix 64 x 64, FOV 192 x 192 mm). The first seven and the last volume in each session were treated as dummy disc acquisitions. To improve localization, a sagittal T1-weighted 3D Magnetization Prepared Rapid Gradient Echo (MPRAGE) volume was acquired (TR=2000 ms, TE=3.9 ms, Flip angle= 7°, matrix=128 x 128, FOV 256 x 256 mm).

Preprocessing and analysis of fMRI data was performed using the SPM2 software. All functional volumes were realigned to the first volume, and the anatomical image was co-registered to the mean functional image to ensure that they were aligned. Images were then spatially normalized, re-sampled at 2x2x2 mm and smoothed, using a 6 mm full width-half maximum (FWHM) isotropic kernel. Data was high-pass filtered using a cut-off value of 128 s and the AR(1) function as implemented in SPM2 was applied. The model was built by convolving boxcar functions for the onsets of the two block types (“faces” and “figures”), with a canonical haemodynamic response function. Individual contrast images were created by subtracting “figures” from “faces”. The contrast images were converted to matrices and submitted for further analyses.

5.5 SUMMARY STATISTICS OF BD GWAS

Summary statistics is considered the net result of a GWAS and is a table containing statistics for each SNP like chromosome number, physical position, allele frequency, odds ratio and p-value for all SNPs. In paper I, collaborators provided summary statistics for TOP [175] and a German GWAS of bipolar disorder [176], as well as the publically available WTCCC bipolar GWAS [82]. In paper III we obtained the significant genes using the cmFDR method of a large and currently unpublished GWAS of BD, from collaborators in San Diego, USA.

5.6 STATISTICAL ANALYSIS

Briefly, Spearman rank correlation coefficient is used in paper I. principal component analysis (PCA), linear regression, Wald test and random permutation test are used in paper II, while Fisher's exact test is used in paper III. Paper I do not access significance, while permutation based methods to assess significance and adjusting p-values for multiple testing is used in paper II, while in paper III we use Fisher's exact test and false discovery rate [184].

5.6.1 A NEW TOOL FOR ASSIGNING SNPS TO GENES

In this study we used the Spearman's rank correlation coefficient of summary statistics (SNPs and p-values) to compare an overall descriptive trend in increased concordance between studies when using LD together with physical distance to assign SNPs to genes. Spearman rank correlation coefficient is closely related to the Pearson's rank correlation coefficient, but uses ranks instead of raw values. It permits the comparison of non-linear variables, and if the ranks within two variables are identical, results in a positive Spearman rank correlation coefficient of 1. A requirement is that the two variables are monotonic, that is there must be a linear or inverse linear relationship between the ranks of the two variables. Calculating Spearman's rank correlation coefficient, without ties, is done with the following formula, where d_i equals the squared difference in rank between each observation of the two variables, and n equal the number of observations. In paper I we used R to calculate the Spearman's rank correlation coefficient between the ranked gene lists returned from three GWAS studies of BD, before and after LD inclusion.

$$\rho = 1 - \frac{6 \sum d_i^2}{n(n^2 - 1)}$$

5.6.2 *APPLYING GENE SET ANALYSIS ON A GWAS USING FMRI ENDOPHENOTYPES*

In this study we used several statistical methods: PCA, linear regression, Wald test and random permutation tests. First, we reduce the fMRI data using PCA [185, 186], which return eigenvectors for each individuals for several eigenvalues. PCA is a mathematical technique that aims to compress or reduce data without loss of (too much) information. The method uses the following steps: a) calculate mean within the samples b) subtract the mean within the samples c) calculate the correlation matrix between the samples. Individual samples are the collection of voxels within each individual and we aim to construct new images which represent the majority of variance between individuals. d) Eigenvectors and eigenvalues are calculated using linear algebra from the square covariance matrix. Then the new "samples" which have eigenvalues above the level of noise, visualized in a scree plot [187], are identified, and the eigenvectors within are used as quantitative traits in linear regression and Wald test to access the significance of SNPs in GWA analysis. Detailed examples of PCA on microarray data can be found in [188] and [189].

First linear regression is used to estimate the regression coefficients of SNPs with the quantitative trait (eigenvectors). The regression coefficients are then used in the Wald test, which compares the difference between a proposed variable of interests (quantitative trait or θ_0) with the maximum likelihood estimate (regression coefficients or θ) divided by the standard error of the likelihood estimate (θ), compared to the normal distribution.

$$\frac{(\theta - \theta_0)^2}{se\ (\theta)}$$

This yields a p-value for each SNP, which is assigned to genes and gene sets. To access the significance of gene sets, we performed random permutation test by sample randomization and re-running the regression and Wald test 100,000 times, using the number of significant genes within the gene sets, weighted by the importance of the eigenvalue as test-statistic, and significance calculated as the number of times the null distributions have equal or greater test statistic (t) then the original test-statistic (T), divided by the number of permutations(n). Both the numerator and denominator are added by 1 to avoid p = 0.

$$p = \frac{t \geq T + 1}{n + 1}$$

We used R to perform the PCA analysis (“princomp”), PLINK for regression and Wald test and to generate the null distributions [190]. Both linear regression and Wald test are executed by the “—assoc” function, if PLINK detects a quantitative trait. We made a custom R function to weight and access the significance of gene sets.

5.6.3 EVIDENCE OF OESTROGEN RESPONSIVE GENES IN BIPOLAR DISORDER

In the last study we use the Fisher’s exact test implemented in “topGO” to test if any GO terms are over-represented among protein interacting- and BD associated genes. Fisher’s exact test is frequently used to test differences between categorical data, where the data are summarized in 2x2 contingency tables. The test assumes that the two groups are independent and mutually exclusive, and yield exact p-values. Here we make use of the “weight” function within “topGO” to increase the score of children nodes within the GO hierarchy: significance scores of connected nodes (a parent and its child) are compared in order to detect the locally most significant terms in the GO graph. This is achieved by down-weighting genes in less significant neighbours [191].

5.6.4 RANDOMIZATION TO ACCESS SIGNIFICANCE

Permutations is a randomization procedure to generate false data, also called null distributions, and compare a test statistic from the original data to the test statistic in the false data in order to determine statistical significance, originally proposed by Fisher in 1935. Ideally all possible permutations (combinations) should be performed, but this is often practically impossible for larger data sets, so an approximation is needed to perform a fixed but large amount of permutations, proposed by Dwass in 1957 [192].

Permutation based methods is a class of non-parametric tests which is advantageous when there is reluctance to make assumptions about the distribution of the data, have good flexibility, guarantee to control the false positive rate (type I errors) [193] and is considered the gold standard for adjustment for multiple testing [162, 194]. Permutation based methods have three requirements a) a null hypothesis b) a test statistic c) null distribution generated by permuting (with replacement) the original data. One critical point is that the null distributions must be permuted in such

a way that confounding factors influencing the original data are also present in the null distributions. For instance randomizing the individual disease status in a GWAS retains several confounding factors which influence the significance of genes like LD, gene length and SNP coverage. Randomizing the significance of genes, can create skewed null distributions, see figure 1. Given a hypothesis, a test statistic and null distributions, a p-value can be easily calculated as the number of times the permuted test statistic is equal or exceed the test statistic in the original data divided by the number of permutations. For instance if the test statistic in the null data is equal or exceed the test statistic in the original data, 10 out of 1000 times, yields a p-value of 10/1000 = 0.01. Often the numerator and denominator are added by 1, to include the original data and to avoid p = 0, important when adjusting for multiple testing [192]. Generating all possible permutations yield exact p-values, while permuting a fixed number of permutations due to practical considerations is known as Monte Carlo permutation tests or random permutation tests, yield approximate p-values [192]. Random permutation tests are used in paper II and paper III.

5.6.5 CORRECTION FOR MULTIPLE TESTING

When testing a hypothesis, a significance level must be defined and by convention, a null hypothesis can be rejected if we can do so with less than 0.05 or 5% probability of being wrong. However, modern genetic and molecular biological methods allow the measurement of hundreds of thousands of elements, resulting in the testing of an equal amount of hypotheses. At a significance level of $p<0.05$, approximately 5% of the elements would be called significant by chance, resulting in thousands of significant findings, thus methods to adjusting p-values for multiple testing are obligatory in statistical inference of high throughput experiments like GWAS, also known as control of the type I error rate [195]. This can be done in several ways, but control of the family-wise error rate and false discovery rate are the most common methods.

The most conservative method is to control of the family-wise error rate (FWER), defining the level of type I error rate at the probability of at least one type I error. Two general branches for control of the FWER exists, single step (same adjustment to all) and sequential (rank based). Example of single step control of FWER is the Bonferroni adjustment, where type I error rate is simply defined as 0.05 / number of tests. Sequential adjustment for control of FWER includes Holm [196]. FWER control

of type I error rate guards against any false positive findings. If using permutations it is possible to make use of the null distributions in controlling type I errors as permutation based FWER control of type I error rate would mean treating each of the permutations as original data and count the number of times any hypothesis in all permutations is equal or exceed the permuted p-value. This approach is also known as the minimum p adjusted p-value [197], and is considered conservative [138] (paper II).

Less conservative than FWER, but arguably more powerful, is control of the false discovery rate (FDR) [184]. High throughput experiments are often said by be hypothesis free, a substrate for formulating new hypothesis, thus a degree of false positive findings may be found acceptable. Control of type I error rate using rank based (FDR), is common, especially in gene expression studies, accepting a proportion of false discoveries among the total rejected null hypothesis. In paper III we adjust the p-values returned from a Fisher's exact test by the Benjamini-Hochberg FDR procedure.

5.7 SOFTWARE

The studies within this thesis have only used open-source software.

R / CRAN R (The Comprehensive R Archive Network) is a programming language and environment and is used throughout all papers in this thesis. Numerous R packages have been used including: IRanges [198] and HDF5 [199], princomp, miscTools [200], rgl [201], misc3d [202] and GenomicRanges [203], biomaRt [204], stringr [205], iRefR [206] and Cairo [207].

cran.r-project.org

PLINK is a command line software, frequently used to perform quality control and association analysis in GWAS [190]. Specifically PLINK was used in quality control of the TOP genotypes (associated papers), and association analysis in (paper II).

pngu.mgh.harvard.edu/~purcell/plink

Affymetrix power tools (APT) APT is a command line collection of functions and algorithms tailor made for the analysis of cross platform Affymetrix GeneChip® arrays, and was here used to call the discrete genotypes of SNPs from raw data,

using the birdseed algorithm (associated papers).
affymetrix.com/partners_programs/programs/developer/tools/powertools.affx

5.7.1 SOURCES FOR GENE SET DATA

As knowledge about genes is accumulating, so do the number of databases, with various biomedical scopes. Unifying these databases into single resources is convenient and helpful in order to conduct comprehensive analysis of high-throughput experiments like GWAS. Here, two unifying resources have been used. In paper II we use the MSigDB database [138] (version 3.0), a collection of gene sets including data from Gene Ontology, KEGG, Biocarta and Reactome. In paper III we make use of protein interaction data, unified in the iRefIndex database (version 6.6.2013), which rely on numerous protein-protein interaction databases including BIND, BioGRID, CORUM, DIP, HPRD, InnateDB, IntAct, MatrixDB, MINT, MPact, MPIDB, MPPI and OPHID [208].

6 SUMMARY OF RESULTS

All papers relate to the biological interpretation of GWAS results, applied to BD and an endophenotype. Paper I elucidates the effect of adding information from linkage-disequilibrium between SNPs returned from GWAS of bipolar disorder (BD) when assigning SNP to genes, in collaboration with the University of Bergen. In Paper II we apply a permutation-based, gene set enrichment method to test the effect of SNPs on brain activation patterns during a functional MRI experiment. Finally, in paper III we make use of protein-protein interaction data to construct a protein interaction network of BD risk proteins and test, if any gene sets are over-represented among protein interacting genes associated with BD.

6.1 A NEW TOOL FOR ASSIGNING SNPS TO GENES

Linkage-disequilibrium-based binning affects the interpretation of GWASs

Down-stream analysis of GWA results and gene set enrichment methods are critically dependant on how SNP are assigned to genes. Typically SNPs within the gene boundaries and neighbouring SNPs, defined by an arbitrary physical distance, are used to assign SNPs to genes. We argue that using physical distance alone is too simplistic as linkage disequilibrium (LD) between SNPs can span over non-negligible distances.

As proof of principle we measured the rank correlation of results between three GWAS sub-studies of BD, using positional and LD based binning. We reported that, by including LD, the mean pairwise rank correlation between studies increases from 0.03 to 0.06, suggesting that including LD results in an overall increase in agreement between studies. We also showed that by including LD, the global SNP coverage increases and that genes without any SNPs using positional binning alone, are included by using LD.

We implemented this method in a flexible R package, denominated “LDsnpR”, which is computational efficient and uses the pairwise linkage disequilibrium (LD) between SNPs pre-calculated from HapMap CEU r27 reference panel, to assign SNP to genes based on distance and LD. In addition, we implemented several methods for calculating aggregate gene scores.

6.2 APPLYING GENE SET ANALYSIS ON A GWAS USING FMRI ENDOPHENOTYPES

Pathway analysis of genetic markers associated with a functional MRI faces paradigm implicate polymorphisms in calcium responsive pathways

SNP genotypes can be combined with brain imaging data to possibly reveal SNPs which influence brain function. However, testing each SNP (~500,000 SNPs) versus each voxel (mm^3) (~200,000), results in many statistical tests (here 0.1 trillion tests) with the following multiple testing issues. Both data types contain a considerable degree of correlated measurements and data redundancy.

We applied principal component analysis on a functional MRI experiment of a negative faces task, and used the most important components as quantitative traits in a GWAS. SNPs moderately associated ($p < 1 \times 10^{-4}$) with the experiment are used in gene set analysis. We permuted the gene set analysis 100,000 times, by sample randomization, in order to asses significance and adjust the retuning p-values for multiple testing using strict permutation based FWER.

The most significant gene set was related to post-NMDA receptor activation events, although there was no statistical significance after strict adjustment for multiple testing. We combined the SNPs within the most significant gene set to localize a single with a peak effect in the inferior frontal gyrus, a region involved in risk aversion and language production.

6.3 EVIDENCE OF OESTROGEN RESPONSIVE GENES IN BIPOLAR DISORDER

Network-based gene set enrichment of genomic regions associated with bipolar disorder reveals susceptibility genes responsive to oestrogen stimulus

Increasing sample size and applying Bayesian statistics in GWAS yield more statistically significant SNPs associated with BD. Here, we conduct a protein interaction analysis of significant SNPs (n=250) identified by a Bayesian method using the summary statistics of PGC GWAS of BD (n cases=15,795).

Cellular processes are the collective result of protein functions, often organized in distinct modules, consisting of groups of physically interacting proteins. Genetic disorders are manifestations of perturbing such functional modules. We make use of protein-protein interaction data to test if susceptibility genes for BD encode proteins

that engage in protein-interactions with other BD susceptibility genes, and if they share a biological context represented by Gene Ontology terms.

We construct a protein-protein interaction network using high confidence protein interaction data and identify one biological process, response to oestrogen stimulus, as significantly over-represented within protein interactions between BD associated genes.

7 DISCUSSION

7.1 A NEW TOOL FOR ASSIGNING SNPS TO GENES

Here we showed that using information of LD between SNPs in a reference panel, together with physical distance, improves SNP to gene annotation. Furthermore, in the process we developed an R package, “LDsnpR”, for this purpose. More specifically, the benefits of using LDsnpR are: a) a framework for assigning SNPs to genes using physical position and LD b) to calculate aggregate gene score based on annotated SNPs c) to increase the genomic coverage by assigning SNPs to genes that do not have any SNPs within its physical boundaries, thus “rescuing” genes. This resulted in the annotation and “rescue” of ~7% of all genes. d) To increase genomic density, that is, more SNPs are assigned to genes (on average and increase from 5.6 to 8.4 SNPs pr. gene). We argue that this gain of information is fast, cheap and free and demonstrate the usefulness of this method as the gain in the agreement or concordance between GWAS. The three studies of bipolar disorder showed an increase in concordance of ~3% of the rank correlation of results, compared to only positional based SNP to gene annotation.

Other studies have also developed methods for SNP to gene assignment using LD. This includes a study which describes a Perl script “ProxygeneLD” [144] and another study that describes an algorithm, which is currently unavailable “LDspline” [145]. The method described in this study, LDsnpR, is faster, more flexible and more user friendly than the two other previous methods. LDsnpR also include calculation of aggregate gene scores.

There are some limitations to this study. The main limitation is that we use underpowered GWAS of bipolar disorder to demonstrate an increase in concordance [118]. The increase in concordance is also quite modest (3%). Another limitation is that there are few, but relatively large LD blocks in the human genome, including the MHC region of chromosome 6, which by using LDsnpR naively, would result in the best scoring SNP within the large LD blocks in this region being assigned to numerous genes, resulting in an overall increase in type I errors (FP).

The findings in this study have been criticized [209]. The opposing argument is that the increase in concordance between studies is due to correlations caused by non-random LD patterns, and not the same SNP signals. This is also a critique of aggregate gene scores of GWA results in general. Although this might be true, the systematic interpretation of GWAS requires SNP to gene assignment, but which approach is more appropriate is subject to discussion [141, 210, 211]. The current consensus in SNP to gene assignment seems to be simple but effective: minimum p-value of SNPs within or close to genes (+- 20 kbps). This simple “rule” is based on findings from eQTL studies, where SNPs influencing gene expression, tend be located quite close to the affected gene [142, 212]. The methods GATES [213] and VEGAS [214], which aggregate and permutes gene scores using LD patterns, is emerging as a gold standard in aggregating SNPs to genes. These algorithms could be implemented in future versions of “LDsnpR”.

LDsnpR is an efficient tool for assigning SNP to genes, with the aforementioned benefits. However, SNP imputation [125], computationally inferring the SNP states of un-genotyped data is now routinely performed in GWAS, making the increase in genomic coverage and density argument provided by “LDsnpR”, obsolete. Nonetheless, the main purpose of “LDsnpR” is to provide a framework for SNP to gene annotation and to calculate aggregate gene scores, based on physical distance and LD, and is still useful even used on imputed SNP data. Another method, GATES, takes into account LD patterns when calculating gene based statistics, and yield an overall increase in significant findings from GWAS studies [213]. This approach could be implemented into LDsnpR to increase its versatility.

7.2 APPLYING GENE SET ANALYSIS IN A GWAS USING FMRI ENDOPHENOTYPES

In this study we developed a new method in an attempt to detect SNPs and associated gene sets possibly influencing brain function during a particular functional MRI (fMRI) experiment. The rationale behind this project was to use methods to improve statistical power when combining SNP and brain imaging data. This was done on two levels, data reduction of fMRI data and gene set analysis of associated SNPs. Data reduction lowers the multiple testing burden and gene set analysis increases power to detect moderate, but recurring signals in related genes. We applied a computationally intensive method, permuting sample labels and re-running

association analysis 100,000 times using five phenotypes, to assess significance by permuted p-value and strict adjustment for multiple testing. The main conclusion of this project was that we were not able to detect any statistically significant gene sets which influenced brain function in this particular fMRI experiment. We were, however, able to identify a biological relevant gene set, “post NMDA receptor activation events”, which seemed to localize a relevant area in the brain, left inferior frontal gyrus, although this is somewhat speculative.

In this study we used principal component analysis (PCA) of a specific fMRI experiment, and treat the returning eigenvectors as endophenotypes in a GWAS. Using PCA, it is common practice to evaluate the returning eigenvalues in Scree plot, also called The Cattell scree test [187], and keep the eigenvalues above the level of noise. In our case the first five components were above the level of noise, capturing 25% of the variance in the experiment. Thus, the majority of information using this approach is lost, an indication of the large individual variability in fMRI experiments, which is a major limitation of this study.

This study was underpowered (n= 246) to detect the small effects of SNPs, but we attempt to remedy this using techniques to improve statistical power. The sample consisted of healthy controls as well as subjects diagnosed with BD. Although there is a large interest in detecting characteristic brain activation signals in BD [215], there is no consensus that such signals exist, although several patterns both in terms of structural as well as functional areas have been found [216-218]. We reasoned that if we were able to detect any significant findings using all subjects, this would encourage us to further formulate diagnosis specific hypothesis. As we were unable to detect any statistically significant findings using all subjects, we reasoned that we would probably not find any significant findings using BD, as this would result in a reduction of sample size. There is a large consensus that brain activity measured by fMRI shows a large degree of variability between individuals and care should be taken when designing, analysing and interpreting results [219]. Several sources of variance in an fMRI experiment are well known like anatomy, cranial shape, head motion, task paradigm, cognitive strategy, medication, physiological noise like heart rate, scanner noise, data analysis method, age, gender, sex and genetics [220]. In this study we do not consider nor adjust for any of these sources of variability, which may obscure genetic associations.

To our knowledge this is the first study performing rigorous gene set analysis using global activation patterns from functional MRI experiment as endophenotypes and testing these against genome wide SNP markers. Several studies have however identified the possible influence of genes using similar approaches including, a genome-wide and whole brain association study, but without gene sets analysis, using parallel ICA of an oddball fMRI experiment (n=63) [221] and a study using candidate genes and structural MRI with subsequent gene set analysis (n=278) [222]. However, fMRI studies are characterized by small sample sizes [223], a consequence of the labour intensive effort in collecting fMRI data, suggesting that most studies, including our own, are underpowered to detect the small effects of individual SNPs [47]. The field of imaging genetics are collecting larger and larger sample sizes attempting to identify significant SNPs [224, 225], primarily using structural MRI (sMRI) as endophenotypes. One of the first GWAS in imaging genetics measured the temporal lobe volume by sMRI in 742 Alzheimer's disease patients, found no significant findings ($p < 5 \times 10^{-8}$) [102], while the largest imaging genetic study ever performed (2012) (n=7795) used sMRI measurements of hippocampal and intracranial volume and found two significant SNPs [224]. Imaging genetics is the convergence of advances in genetics and brain imaging, fields which by themselves generate large amounts of data [226]. When combined, computational and statistical issues rise, complicating the effort in elucidating possible common genetic effects on brain structure and function, and new statistical methods are under development to handle the amount of correlated tests [227, 228].

7.3 EVIDENCE OF OESTROGEN RESPONSIVE GENES IN BIPOLAR DISORDER

In this study we use known protein interactions as a logical filter to test for over-represented Gene Ontology (GO) terms among protein encoding genes forming interactions with other protein encoding genes associated with BD.

This study is based on the results of on a currently un-published study (Jan 2014) using the cmFDR methodology [229, 230] on the most recent PGC GWAS of BD (n cases = 15,795 and n controls = 22,365). The identified SNPs (n=250) were assigned to 501 protein encoding genes. Between these 501 proteins we identified 43 protein interactions. While this network may be of qualitative interest by itself, we test if these proteins share any biological context using Gene Ontology terms using

classical over-representation methods. We found the terms “response to oestrogen stimulus” significant after adjustment for multiple hypothesis testing.

This is not the first time hormonal activity has been associated with BD. A study testing for enrichment of GO terms in the WTCCC BD GWAS, reported hormonal activity as the best scoring GO term [166]. Fluctuations in oestrogen levels during menstrual cycles, after childbirth and menopause have been associated with affective episodes and vulnerability to develop depression in females [231, 232]. Women with mood disorders have increased risk of experiencing acute episodes after childbirth [15928351, 23247604], which may suggests a hormonal influence. Although BD is equally prevalent in both genders, rapid cycling BD is more common in women. Tamoxifen, a selective oestrogen receptor modulator, primarily used in therapy for hormone receptor-positive breast cancer in pre-menopausal women, have been shown to be effective in the treatment of mania [233-235], where the proposed mechanism is its ability to inhibit protein kinase C. Oestrogen (estradiol) has been shown to activate protein kinase C in a variety of cell types including neurons [236]. Activation of membrane bound oestrogen receptors in hippocampal neurons trigger calcium mediated signalling where neurons expressing membrane bound oestrogen receptor, also co-express the L-class of calcium channels [237, 238], which includes the prime BD susceptibility gene CANCA1C [39, 239, 240]. This might suggest that dysregulation of estradiol activated and calcium mediated signalling, and/or activation of PKC by estradiol in BD might be one molecular mechanism in the etiology of BD. It remain unclear if estradiol levels are abnormal in individuals suffering from BD or if women BD are more sensitive to fluctuations in estradiol levels [241]. The finding in this study is based two layers of knowledge, known protein interactions and known gene functions. Thus bias is introduced towards well studied proteins, a general issue in gene set enrichment. Further the list of BD associated genes is massively reduced from 501 associated protein encoding genes from to 43. This is a considerable loss of candidate genes. Nonetheless we were able to pinpoint one possible biological process which might contribute to the underpinnings of BD etiology.

7.4 STRENGTHS AND LIMITATIONS

The main limitation of all studies is statistical power. The first study use underpowered GWAS of BD to demonstrate an increase in concordance between

studies. The second study is a GWAS of a small sample (n=247) using fMRI as an endophenotype and the third study we include nominal significant SNPs. The second and third study should be considered as a hypothesis generating study and which encourages further validation. However, the main strength of the second and third studies is that we do not make biased assumptions of single candidate gene or regions of interest. Although these biased assumptions would reduce the multiple testing burden, and increase the statistical power, applying such constraints on whole-genome or whole-brain data are generally not recommended [242, 243].

The field of psychiatric genetics rely on the symptomological classification of mental disorders. Defining a quantitative variable in psychiatric genetics would potentially increase the statistical power to detect significant findings by adding more phenotypes into a single GWAS resulting in an increased sample size, similarly as the combined case-control GWAS of five mental disorders, identifying five significant findings [244]. A putative study of the same sample using a biomarker or a quantitative trait is hampered by the absence of any such biomarkers or missing quantitative traits in psychiatric genetics.

Gene set analysis relies on current biomedical knowledge of genes, and is limited by the current understanding of gene functions and how this knowledge is re-phrased and annotated in databases. The nature of biocuration makes it lag behind emerging experimental knowledge as indicated in by approximately a hundred genes annotated with GO term “postsynaptic density” while proteomic studies have identified over a thousand proteins localized to the postsynaptic density [245, 246]. Gene set analysis used in the second and third studies, has several other general limitations including: overlapping gene sets resulting in issues in multiple testing, low resolution in annotation i.e. gene sets terms or gene sets tend to be quite generic and the inability to implement or capture the dynamic properties of biological processes, in particular cell specific information [247].

There is no methodological gold standard in gene set analysis and multiple steps such as assigning SNPs to genes, different sources of gene sets, database versions and different methods to assess statistical significance are sources of variability [248]. Although gene set analysis methods have provided invaluable insights in numerous studies including GWAS [247, 249] and is routine in interpreting high

throughput experiments, the methods has provided somewhat inconclusive results when applied to GWAS results in psychiatric genetics [250]. This may be a consequence of the current understanding of SNPs and the large gap between influential SNPs and reported statistical significant SNPs.

7.5 CURRENT LARGE SCALE GWAS IN BIPOLAR DISORDER

The Psychiatric GWAS Consortium, formed in 2010 [116], has conducted the largest GWAS of bipolar disorder (BD) to date which includes the TOP sample. The GWAS of BD (n cases = 11,977) resulted in four significant findings associated with the genes CACNA1C, ODZ4 and two larger regions covering C11orf80 and near DHH. A combined GWAS of schizophrenia and bipolar disorder yielded additional significant findings in CACNA1C, ANK3 and the ITIH3-ITIH4 region [134]. These overlapping findings between schizophrenia (SCZ) and BD have been further refined [244]. Gene set enrichment of genes associated with bipolar disorder from the WTCCC study, suggested the influence of hormonal processes in BD [166], a result that is similar as paper III in this thesis. However, it is difficult to assess the impact of gene set enrichment as these findings as it is based on non-significant SNP associations. None of these aforementioned GWAS have successfully identified any conclusive biological processes or distinct molecular contexts which are perturbed in these disorders, although “examination of candidate genes at these loci suggests the involvement of neuronal calcium signalling” [251]. Polygenic analysis of the degree of liability conferred by SNPs concludes that 25% of the estimated heritability is explained by common genetic variation [252], suggesting further increase in sample size would yield more significant findings and the application of novel statistics methods to increase the number of associated SNPs.

7.6 CURRENT UNDERSTANDING OF COMPLEX DISEASES

This thesis is performed in parallel with advances in human genetics and an on-going debate of the usefulness of GWAS [253-256]. During the five year window from the WTCCC study in 2007 [82] to the largest GWAS of date of blood lipids in over 200,000 individuals in 2011 [257], several insights have emerged. SNPs are highly influential in some complex diseases, in particular, auto immune diseases like systemic lupus erythematosis, multiple sclerosis, type I diabetes, and rheumatoid arthritis [254]. More specifically GWAS have highlighted biological processes like: autophagy in Crohn’s disease, IL-23R pathway in rheumatoid arthritis and factor H in

age-related macular degeneration [256]. However, most GWAS of complex diseases have not managed to identify the expected number of significant findings indicated by the explained heritability of significant findings. Initially this was attributed to the small effects of SNPs in general, a need tighten the phenotypic definitions as well to increase sample sizes. This inability to identify the bulk of the expected genetic susceptibility sparked the missing heritability debate [258, 259], where numerous possible factors explaining “the missing heritability” of complex disease have been proposed including epistatic effects between SNPs, increased attention towards epigenetic mechanisms, rare variants and de-novo events as well a call for more sophisticated statistical tools [259-262]. Although statistically significant SNPs only explain a small fraction of the estimated heritability, remarkably, a large proportion of the estimated heritability is explained by all SNPs, were chromosome length is linearly related to explained heritability, suggesting the influence of thousands of SNPs in SCZ and BD [250].

In summary, GWAS have revealed substantial insights in human genetics including a) many loci contribute to complex traits b) SNPs are associated to several traits c) considering all SNPs (not just the significant ones) explains a large amount of additive genetic variation d) SNPs with large effects are almost non- existent [256].

8 FUTURE PERSPECTIVES

Genes carry information of how biomolecules interact with the molecular environment. Most genes encode proteins, with the evolutionary adaptive purpose to interact directly or indirectly with other proteins and biomolecules. The number of interactions can sometimes be very high. As such, disease is the organismal manifestation of dysfunctional or abnormal interactions between genes in specific cellular contexts. Epistasis [263], the non-additive statistical dependence between SNPs, or modifier genes of a trait, has been suggested to explain some of the observed “phantom” heritability in GWAS [264, 265].

Many monogenic conditions show incomplete penetrance and variable expressivity of traits, where the observable symptoms of the conditions may vary greatly [266]. The variable expressivity between individuals is thought to be caused by genetic variations of modifier genes within individuals. The influence of modifiers genes in

complex diseases may be identified using SNP data. However, the exact combinations of modifies genes within individuals is not taken into account in conventional GWAS. Hypothetically, individuals suffering from BD may not share a universal and common disease mechanism, but possibly several molecular dysfunctions may lead to disease, analogously to the many molecular mechanisms which may give rise to cell proliferation and cancer. Studies have shown that neurological diseases, together with cancer, are genetically heterogeneous and form highly interconnected protein networks [254, 267]. For instance the intracellular matrix forming the post-synaptic density region, have been shown to be composed of over 1,000 proteins [246]. Obviously this structure may dysfunction in numerous ways. As such, individuals suffering from a complex disorder like BD may contain several, but distinct combinations of modifier genes or codes of epistatic interactions, information which is lost during the single marker approach of conventional GWAS, which treat each SNP as independent entities across a large number of individuals. Statistical testing the dependence between every SNP within every individual is a considerable computational task, which may be facilitated by data reduction techniques like haplotype phasing, resulting in testing haplotype-haplotype interactions within affected individuals, and subsequent gene set analysis of epistatic interactions. A recent study using identifying epistatic interactions and subsequent gene set enrichment in BD found “enrichment of genes in the cadherin, Wnt and axon guidance signalling pathways is suggestive of a developmental origin for BD” [268].

The amount of data generated by modern genetics is enormous, and the brute force approach of GWAS including SNP imputation in elucidating the genetics of complex human diseases also seems to be its Achilles' heel. The number of hypothesis tests is large, even larger in imaging genetics, and as statistical methods to adjust for multiple hypothesis testing require independence, results in a substantial amount of type II errors. Many consider permutation based methods as the gold standard in statistical inference. However, the reluctance to share raw GWAS data together with computational practicalities, makes permutation based methods and sample randomization rare in GWAS.

GWAS of BD disorders have revealed the influence of ten thousand SNPs [244]. However, the inability to identify converging and distinct disease mechanisms,

including through gene set analysis, seems to be caused by the absolutely tiny effects of a very large number of SNPs related to genes which participate in several biological processes, a scenario the current gene set analysis methods are not developed to take into account. In hindsight, it may not be very surprising that SNPs with large disease causing effects do not exit, as evolutionary forces would weed out such alternations before it became distributed within a population.

Even though all SNPs substantiate a considerable degree of the estimated heritability in BD, the majority of the estimated heritability remains unaccounted for and may reflect the importance of other mechanisms proposed during the missing heritability discussion including epistatic interactions between genetic variants, gene and environment interactions, improve definitions of phenotypes, elucidate effects of sex chromosomes and increase in focus on other genetic alterations like rare variants, de-novo mutation and epigenetics [259]. Paternal age have shown strong associations with the disorders autism and schizophrenia [269], and given the overlapping susceptibility demonstrated by GWAS and shared clinical symptomology between SCZ and BD, paternal age may also be associated with BD. A very recent study showed an increased hazard ratio between offspring of young and old fathers, where children of older fathers have an increased hazard ratio of 24 for developing bipolar disorders [270]. This may emphasize the importance of de novo mutations in forming susceptibility to severe mental disorders.

9 CONCLUSIONS

In this work several approaches have been used in an attempt to better appreciate GWAS results in general and BD in particular. The first study attempts to improve methods to facilitate the non-trivial task of assigning SNP to genes and has broad applications, although the specific gain in BD research is limited. In the second study we attempt to identify biological related genes which are associated with neural activation patterns, and represent a novel analytical method in brain imaging genetics in general. However, as we were unable to detect any significant findings, and we decided to not test BD specific hypothesis further, as this would result in reduction of sample size and further loss of statistical power. In the last study we make use of protein interactions between BD risk genes, in an attempt to identity any over-represented biological process among protein interactions between BD risk genes, resulting in a significant over-representation of oestrogen responsive genes. The last study raises a hypothesis which is clinically relevant to mood disorders and further experimental work could relatively easily determine oestrogen levels during mood cycles in individuals with BD as well as controls, which is currently not well described in the scientific literature. However, six genes are causing the enrichment of oestrogen, and polygenic analysis suggests the influence of thousands of SNPs, and as such, the oestrogen hypothesis may represent one of many processes involved in forming susceptibility to BD.

10 REFERENCES

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